THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

HIF1 is a regulator of hematopoietic progenitor and stem cell development in hypoxic sites of the mouse embryo

Citation for published version:
Imanirad, P, Solaimani Kartalaei, P, Crisan, M, Vink, C, Yamada-Inagawa, T, de Pater, E, Kurek, D, Kaimakis, P, van der Linden, R, Speck, N & Dzierzak, E 2014, 'HIF1 is a regulator of hematopoietic progenitor and stem cell development in hypoxic sites of the mouse embryo', *Stem Cell Research*, vol. 12, no. 1, pp. 24-35. https://doi.org/10.1016/j.scr.2013.09.006

Digital Object Identifier (DOI):
10.1016/j.scr.2013.09.006

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Stem Cell Research

Publisher Rights Statement:
|Available under Open Access
License: http://creativecommons.org/licenses/by-nc-nd/3.0/

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

OPEN ACCESS

Download date: 06. May. 2023

Stem Cell Research (2013) 12, 24–35

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/scr

HIF1α is a regulator of hematopoietic progenitor and stem cell development in hypoxic sites of the mouse embryo☆

Parisa Imanirad[a,1], Parham Solaimani Kartalaei[a,1], Mihaela Crisan[a,1], Chris Vink[a], Tomoko Yamada-Inagawa[a], Emma de Pater[a], Dorota Kurek[a], Polynikis Kaimakis[a], Reiner van der Linden[a], Nancy Speck[b], Elaine Dzierzak[a,*]

[a] *Erasmus MC Stem Cell Institute, Department of Cell Biology, Erasmus Medical Center, Rotterdam, The Netherlands*
[b] *Department of Cell and Developmental Biology, Abramson Family Cancer Research Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA*

Received 11 September 2013; accepted 17 September 2013
Available online 2 October 2013

Abstract Hypoxia affects many physiologic processes during early stages of mammalian ontogeny, particularly placental and vascular development. In the adult, the hypoxic bone marrow microenvironment plays a role in regulating hematopoietic stem cell (HSC) function. HSCs are generated from the major vasculature of the embryo, but whether the hypoxic response affects the generation of these HSCs is as yet unknown. Here we examined whether Hypoxia Inducible Factor1-alpha (HIF1α), a key modulator of the response to hypoxia, is essential for HSC development. We found hypoxic cells in embryonic tissues that generate and expand hematopoietic cells (aorta, placenta and fetal liver), and specifically aortic endothelial and hematopoietic cluster cells. A Cre/loxP conditional knockout (cKO) approach was taken to delete *HIF1α* in Vascular Endothelial-Cadherin expressing endothelial cells, the precursors to definitive hematopoietic cells. Functional assays show that HSC and hematopoietic progenitor cells (HPCs) are significantly reduced in cKO aorta and placenta. Moreover, decreases in phenotypic aortic hematopoietic cluster cells in cKO embryos indicate that HIF1α is necessary for generation and/or expansion of HPCs and HSCs. cKO adult BM HSCs are also affected under transplantation conditions. Thus, HIF1α is a regulator of HSC generation and function beginning at the earliest embryonic stages.
© 2013 The Authors. Published by Elsevier B.V. All rights reserved.

Introduction

Mammalian development and many aspects of it, such as placentation, vascular development and hematopoiesis take place in a hypoxic microenvironment (Dunwoodie, 2009; Mohyeldin et al., 2010; Semenza, 2012; Simon and Keith, 2008). In the adult, hematopoietic stem cells (HSCs) localize to the bone marrow (BM) which is estimated to have oxygen levels as low as 1% (Chow et al., 2001). Several studies indicate that

☆ This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

* Corresponding author at: Erasmus University Medical Center, Erasmus MC Stem Cell Institute, Dept. of Cell Biology, PO Box 2040, 3000 CA Rotterdam, The Netherlands. Fax: +31 10 7044743.
E-mail address: e.dzierzak@erasmusmc.nl (E. Dzierzak).
[1] These authors contributed equally to this study.

1873-5061/$ - see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.scr.2013.09.006

long-term repopulating HSCs are maintained at the lowest end of the oxygen gradient in the BM (Kubota et al., 2008; Parmar et al., 2007). Analysis of adult BM by the intracellular incorporation of the hypoxia marker Pimonidazole shows that quiescent HSCs are enriched in the highly hypoxic cell fraction (Takubo et al., 2010). Also, *ex vivo* cultures have been shown to maintain and expand repopulating HSC activity under hypoxic conditions (Danet et al., 2003). Thus, the hypoxic response is thought to protect these important stem cells from oxidative stress.

The master regulators of the hypoxic response are Hypoxia Inducible Factors (HIFs). HIFs are heterodimeric transcription factors consisting of HIFα (HIF1α, HIF2α, and HIF3α) and HIF1β subunits (Dunwoodie, 2009; Mohyeldin et al., 2010; Semenza, 2012; Simon and Keith, 2008). HIF1β protein is constitutively present, whereas HIF1α and HIF2α proteins are regulated by cellular oxygen concentration. Under normoxic conditions (>5% oxygen), HIFα proteins are targeted for proteasomal degradation. In situations of hypoxia, the HIFα proteins are stabilized in the cytoplasm, dimerize to HIF1β and translocate to the nucleus where they bind to hypoxia-responsive elements (*HREs*) in the promoter regions of almost 200 hypoxia-targeted genes to regulate their transcription. Most transcriptional responses to hypoxia have been attributed to HIF1α and HIF2α. They not only regulate the expression of many common genes such as *Vegf* and genes of the glycolytic pathway, but also regulate some unique target genes (Keith et al., 2012; Raval et al., 2005). HIF1α is widely expressed *in vivo* and HIF2α is also expressed in a variety of cell types (Wiesener et al., 2003).

Studies in the mouse embryo revealed central roles for HIFs in development. From embryonic day (E)8.5 onwards to E18, stabilized HIF1α protein is detectable in the mouse conceptus (Iyer et al., 1998), confirming that many regions of the growing embryo are hypoxic (Ryan et al., 1998). Germline deletion of *HIF1α* (KO) results in E10.5 embryonic lethality, with a failure in placenta development, abnormal neural fold formation, defective heart and yolk sac vascular development and a smaller dorsal aorta (Cowden Dahl et al., 2005; Iyer et al., 1998; Kotch et al., 1999; Ryan et al., 1998). E9.5 KO embryos show hematopoietic defects: Erythroid progenitor numbers are reduced, BFU-E colonies are not fully hemoglobinized and the levels of *Epo*, *EpoR*, *Vegf* and *VegfR1* mRNA are significantly decreased (Yoon et al., 2006). Similarly, *HIF2α* and *HIF1β* germline KO embryos suffer from early embryonic lethality and show some overlapping multi-organ defects, including vascular and hematopoietic defects. Yolk sac hematopoietic progenitor activity is decreased and hematopoietic cells become apoptotic by E10.5 (Adelman et al., 1999; Maltepe et al., 1997; Ramirez-Bergeron et al., 2006). The vasculogenesis defect observed in E8.5 *HIF1β* KO embryos could be rescued in culture by addition of VEGF protein (Ramirez-Bergeron et al., 2006), suggesting that HIFs regulate the development of vascular/hematopoietic system. This early lethality precludes the study of HSC development. However, the role of HIF1α in the regulation of adult BM HSC function was investigated using a conditional knockout approach using *MX1-Cre:HIF1α*$^{fl/fl}$ mice (Takubo et al., 2010). Absence of *HIF1α* was associated with increased cycling, leading to HSC senescence and exhaustion in serial transplantations.

The first HSCs are generated in the major vasculature (aorta–gonad–mesonephros (AGM), vitelline and umbilical arteries) of the mouse embryo at E10.5 (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996). At this time hematopoietic progenitor cells (HPCs) and HSCs emerge from vascular endothelial cells (Vascular Endothelial-Cadherin expressing; VEC$^+$) (Chen et al., 2009; Zovein et al., 2008) in a process called endothelial-to-hematopoietic transition (EHT) (Boisset et al., 2010) and form hematopoietic cell clusters that line the arterial walls. Since *HIF1α* conditional deletion in adults affects HSCs, we tested whether conditional deletion of *HIF1α* in VEC$^+$ cells would influence HSC generation and/or function. We show here in a *VEC-Cre:HIF1α*$^{fl/fl}$ mouse model that HIF1α regulates HPC and HSC production in the AGM and placenta at midgestation.

Materials and methods

Mice strains, embryo generation and cell preparation

HIF1α$^{fl/fl}$ (Ryan et al., 1998) (Jackson Laboratories) and *VEC-Cre* mice (Chen et al., 2009) were maintained on a C57BL/6 background. To obtain *VEC-Cre/+:HIF1α*$^{fl/fl}$ animals, *VEC-Cre/+* mice were crossed to *HIF1α*$^{fl/fl}$ mice and the resulting *VEC-Cre/+:HIF1α*$^{fl/+}$ offspring were crossed to *HIF1α*$^{fl/fl}$ mice. Genotypes were determined by PCR. Embryo production used the day of vaginal plug discovery as embryonic day 0. Somite pairs were used to stage embryos. All animal procedures were carried out in compliance with Standards for Care and Use of Laboratory Animals.

AGM, YS, PL (fetal), and FL were dissected (Robin and Dzierzak, 2005), collagenase (0.125%) treated for 45–60 min at 37 °C, washed in PBS/FCS/PS (phosphate-buffered saline (Gibco Inc.), 10% heat-inactivated fetal calf serum (GibcoBRL), penicillin/streptomycin) and pipetted to disperse cell clumps. FL dissociation was performed only by vigorous pipetting. PL and BM cells were Ficoll fractionated. Viable cells were counted by trypan blue exclusion and kept in PBS/FCS/PS at 4 °C. Peripheral blood erythrocytes were lysed (BD Biosciences).

PCR analysis

Embryos were genotyped with the KAPA mouse genotyping kit (KAPA Biosystems). PCR was performed with 1 μl DNA in 1× KAPA2G Fast Genotyping Mix and 0.50 μM primers: 3 min denaturation at 95 °C, 35 cycles of denaturation (15 s at 95 °C), annealing (15 s at 65 °C [*WT* and *fl HIF1α* alleles] or 58 °C [*Vav-/VEC-Cre*]) and elongation (10 s at 72 °C). *HIF1α* primers TGCTCATCAGTTGCCACTT and GTTGGGGCAGTAC TGGAAAG yield PCR products of 600 bp (WT) and 650 bp (fl). VEC- (*Cdh5*) or Vav-Cre forward primers CCCAGGCTGA CCAAGCTGAG and GGCGACAGTTACAGTCACAGAAGAGG respectively and common reverse primer GCCTGGCGATCC CTGAACATG yield 300 bp or 500 bp products. *Hif1α* primers TGGGGATGAAAACATCTGCT and TTGATCTTTCCGAGGACCTG were used to detect WT (960 bp), fl (1000 bp) and recombined (Δ; 300 bp) alleles: 5 min denaturation at 94 °C, 35 cycles of denaturation (1 m at 94 °C), annealing (30 s at 59.5 °C) and elongation (15 s at 72 °C), and a final elongation (10 m at 72 °C). *Ymt* primers CTGGAGCTCTACA GTGATGA and CAGTTACCAATCAACACATCAC and *myogenin* primers TTACGTCCATCGTGGACAGC and TGGGCTGGGTGTT AGTCTTA yield products of 342 bp and 245 bp respectively: 5 min denaturation at 94 °C, 29 cycles of denaturation

(1 m at 94 °C), annealing (2 m at 60 °C) and elongation (2 m at 72 °C), and a final elongation (10 m at 72 °C). Fragment intensities were measured by ImageQuant software. For *Ymt* quantitation, the ratio between the *Ymt* and *myogenin* fragments (DNA normalization) was measured against a standard control dilution curve. *Hif1α* recombination quantitation was performed by Δ / (Δ + fl + WT).

Hematopoietic cell assays

In vitro progenitor assay

Cells were plated at various dilutions in triplicate in methylcellulose (MethoCult GF m3434, Stem Cell Technologies Inc.) with 1% PS and incubated at 37 °C, 5% CO_2 for 12 days. Hematopoietic colony types were distinguished by morphology and counted with an inverted microscope. CFU-C colonies were harvested in 0.5 ml lysis buffer for DNA isolation and recombination PCR.

In vivo HSC transplantations

Female, Ly5.1 or C57BL/6 recipient mice (8–20 wks) were irradiated (split dose of 9 Gy γ-irradiation, 2.5 h apart) and cells injected intravenously *via* the tail vein. Donor chimerism was assessed by either Ly5.1/5.2 FACS or semiquantitative PCR on recipient tissues at >4 months post-transplantation. Two embryo equivalents (ee) of AGM cells or 1 ee of PL cells was co-transplanted with 2×10^5 spleen cells (recipient background) to support short-term survival. 2×10^6 spleen cells or 1500 LSK cells were injected into primary recipients and 2×10^6 Ficoll-fractionated BM cells or 600 LSK cells from primary recipients were injected into secondary recipients.

Flow cytometry analysis and sorting

Cells were incubated for 30 m at 4 °C with anti-CD31-PE-Cy7, c-Kit-APC, CD41-PE, Mac1-FITC, Gr1-PE, CD4-PE, CD8-PE, c-Kit-PE, Sca1-FITC, B220-PE, and TER119-FITC antibodies and washed in PBS/10% FCS. Donor cell chimerism was measured with anti-CD45.1-PE and anti-CD45.2-FITC antibodies. All the antibodies were from BD Pharmingen. Dead cells were eliminated by Hoechst 33258 labeling (Molecular Probes). Cell analysis and sorting were performed on FACS-SORP, FACSAria II or III cell sorters (BD Biosciences).

Hypoxyprobe labeling and whole mount embryo staining

C57BL/6 WT pregnant mice were injected with Hypoxyprobe-1 (60 mg/kg; Hypoxyprobe, Inc. Burlington, USA) and sacrificed 45 min later. E10 and E11 embryos were fixed in 4% PFA/PBS (4 °C, overnight), washed twice with PBS, paraffin-embedded and sectioned (7 μm). After deparaffination, sections were boiled (15 min) in 0.01 M citrate buffer (pH 6.0). Endogen peroxidase activity was blocked (10 min with 1.5% H_2O_2/1% BSA/0.05% Tween/PBS). Primary antibody Hypoxyprobe-1 MAb1 (mouse 1:50; Hypoxyprobe, Inc. Burlington, USA) and HRP-conjugated secondary antibody (1:500, DAKO) were used with DAB peroxidase substrate. Sections were hematoxylin counterstained and observed with an Olympus BX40 light microscope.

As described previously (Yokomizo and Dzierzak, 2010; Yokomizo et al., 2012), whole embryos were fixed for 20–30 min in 2% paraformaldehyde/PBS on ice, and dehydrated in 100% methanol. After dissection, embryos were rehydrated, blocked (Vector Laboratories) and stained with biotinylated rat anti-mouse CD31 (MEC13.3, BD Biosciences) and subsequently rat anti-mouse c-Kit (2B8, BD Biosciences) in blocking buffer (PBS/0.4% TritonX-100/1% skim milk) overnight at 4 °C and washed. Secondary antibodies were streptavidin-Cy3 (Jackson ImmunoResearch) and goat anti-rat IgG-Alexa647 (Invitrogen), respectively. Samples were analyzed on a Zeiss LSM 510 Meta microscope.

Cell cycle analysis

BM cells were stained with anti-lineage-PE antibody cocktail. Lin^- cells were sorted and stained with anti-c-Kit-APC and anti-Sca1-PE-Cy7 antibodies. Cells were fixed with 2% paraformaldehyde for 1 h, washed, and incubated overnight in 0.2% Triton. After washing, cells were incubated with anti-Ki67-FITC antibody for 2 h, washed, stained with Hoechst 33342 and analyzed. IgG-FITC was used for the negative control (BD Pharmingen).

Statistical analysis

Data are presented as mean ± SD. Significant differences (p-value < 0.05) were calculated using Student's t-test.

Results

HIF1α-deficiency in VEC-expressing cells affects hematopoietic progenitors in embryonic sites showing *in vivo* hypoxia

To examine whether hematopoietic tissues of the midgestation mouse embryo are hypoxic, pregnant dams were injected with Pimonidazole and embryos (E10 and E11) were examined for Hypoxyprobe staining. Hypoxic cells were observed in the AGM, placenta and fetal liver (Fig. 1). No background staining was found in controls, except for a few cells in the placenta. The background observed in the endodermal cell layer of the yolk sac (due to high levels of peroxidases) prevents proper evaluation of hypoxia in this tissue. The E10 AGM region showed localized hypoxic aortic endothelial cells (arrow) and hypoxic cells in emerging aortic hematopoietic cells and clusters (arrowheads). Not all hematopoietic clusters or cluster cells were hypoxic. Quantitation of hypoxic cells was performed by counting hematopoietic clusters, cluster cells and endothelial cells on transverse embryo (33sp) sections (every 5th section was counted from the forelimb to the hindlimb region): 57 ± 16% of hematopoietic clusters and 40 ± 19% of cells within the clusters were Hypoxyprobe positive. Only 19 ± 13% of endothelial cells were Hypoxyprobe positive. The mesonephric tubules were also hypoxic and in the fetal part of the E10 placenta, we found areas of high level Hypoxyprobe staining that are most likely endothelial cells (arrow). At E11, only very few, low intensity staining hypoxic cells were evident in the aorta and placenta (not shown).

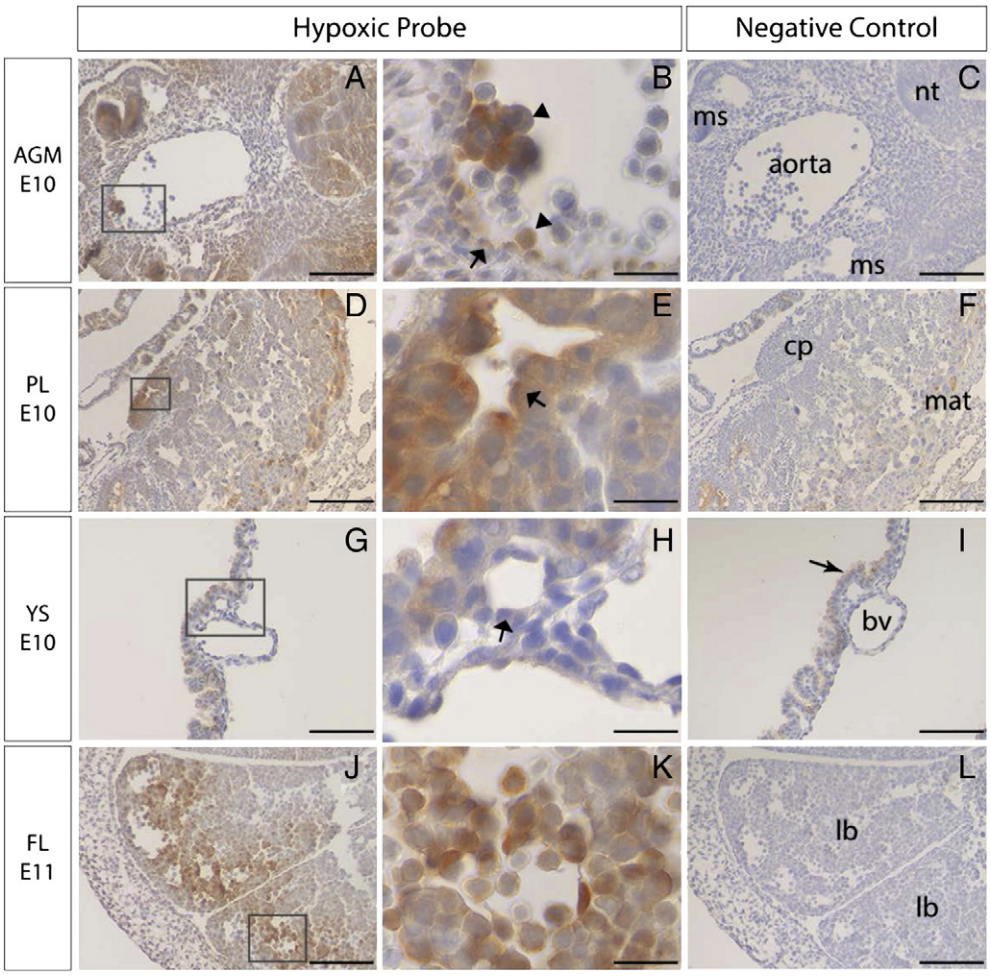


Figure 1 Detection of hypoxic areas of E10 and E11 mouse embryos. Specific Hypoxyprobe (brown) staining shows areas of hypoxia in E10 (33sp) AGM, placenta and YS and E11 (44sp) FL sections (7 μm) of mouse embryos. The presence of hypoxic probe was detected in hematopoietic cluster cells (arrowheads) and some endothelial cells (arrow) in the AGM (A, B), placenta (D, E), FL (J, K) but not in the YS (G, H). Selected rectangles in the first column are shown at greater magnifications in the second column. Negative controls for each tissue staining are shown in the third column. Scale bar: 100 μm (A, C, G, I, J, L), 20 μm (B, E, H, K) and 200 μm (D, F). ms = mesonephros; nt = neural tube; cp = chorionic plate; mat = maternal part of placenta; bv = blood vessel; lb = liver lobe.

However, the E11 fetal liver showed an abundance of hypoxic hematopoietic and endothelial cells (Fig. 1).

To test whether hematopoietic development in these tissues is affected by the response to hypoxia, conditional knock out (cKO) *VEC-Cre/+:HIF1α$^{fl/fl}$* embryos were examined. The VEC-Cre mouse strain we used in these studies was previously shown to affect the recombination and expression of the LacZ reporter in all E10 aortic endothelial and hematopoietic cluster cells and provided evidence that up to 90% of FL cells and 100% of adult BM cells are derived from VEC expressing precursors (Chen et al., 2009). Based on these data, the overlap between VEC expressing cells and Hypoxyprobe positive aortic cells is expected to be complete, thus allowing the examination of the role of Hif1α in hypoxic aortic cells during EHT.

At E9 to E11, *VEC-Cre/+:HIF1α$^{fl/fl}$* cKO embryos exhibit no visible vascular abnormalities, have normal circulation and show no fetal liver anemia. However, hematopoietic progenitor assays showed reductions in total colony forming unit-culture (CFU-C) numbers beginning at E9 in AGM and PL tissues (but not the YS) as compared to WT controls (Table 1, upper panel). At E10 total CFU-C numbers of cKO AGM and PL are significantly decreased (on average 3 and 2.1 fold, respectively) compared to WT controls (Fig. 2a). E10 cKO AGM CFU-MIX are decreased almost 5-fold, and the decrease in all other colony types is significant except for CFU-G (Table 1, lower panel). The numbers of CFU-M and CFU-MIX are also significantly decreased in E10 cKO PL. Only BFU-E were significantly reduced in E10 cKO YS, consistent with previously published data on germline *HIF1α$^{-/-}$* YS (Yoon et al., 2006).

Decreases in total CFU-C numbers of cKO tissues persist at E11, although to a lesser extent. A significant 1.6-fold decrease is observed in AGM total CFU-C numbers (Fig. 2b and Table 1), with CFU-MIX and CFU-M significantly decreased as compared to WT. In the cKO FL, total CFU-C numbers are significantly decreased by 1.6-fold and a significant decrease is observed for cKO PL CFU-MIX, but not for total CFU-C numbers. In most cases, the heterozygous cKO tissues also showed reduced numbers of CFU-C, suggesting a *HIF1α* dosage effect.

Table 1 Summary of CFU-C data for +/+:*HIF1α*$^{fl/fl}$ and *VEC-Cre*/+:*HIF1α*$^{fl/fl}$ embryos. (Upper panel) Hematopoietic progenitor assays were performed E9, E10 and E11 AGM, placenta (PL) and yolk sac (YS) cells and E11 fetal liver (FL) cells isolated from WT (+/+:*HIF1α*$^{fl/fl}$) and cKO (*VEC-Cre*/+:*HIF1α*$^{fl/fl}$) embryos (see Fig. 1). The number of embryos analyzed per genotype, somite pair stages, total CFU-C numbers per tissue (mean ± SD), fold changes of CFU-C numbers obtained from cKO tissues compared to WT controls, and significance of changes are presented here. Significant differences are indicated with asterisks. *: p-value < 0.05, **: p-value < 0.005. (Lower panel) Specific hematopoietic colony types found by methylcellulose progenitor assay of tissues of wild type (WT) and *VEC-Cre:HIF1α*$^{fl/fl}$ embryos.

Tissue	Day	Somite pair group	Number of WT embryos analyzed	Number of CFU-C total/WT tissue	Number of cKO embryos analyzed	Number of CFU-C total/cKO tissue	Fold decrease
AGM	9	20–27	5	4 ± 2.7	4	2.3 ± 3.2	1.7
	10	30–34	3	130 ± 44	4	43.0 ± 20	3.0**
	11	43–47	3	199 ± 35	3	128 ± 20	1.6**
PL	9	20–27	4	14 ± 11	4	5 ± 7	2.8
	10	30–34	4	168 ± 31	7	80 ± 39	2.1**
	11	43–47	3	329 ± 137	3	230 ± 53	1.4
YS	9	20–27	4	196.3 ± 93	4	208 ± 148	1.0
	10	30–34	4	558 ± 154	8	389 ± 134	1.4
	11	43–47	3	479 ± 99	3	360 ± 189	1.3
FL	11	44–46	6	1956 ± 691	6	1236 ± 348	1.5*

Tissue	Day	CFU-type	WT	cKO	Fold decrease	p-Value
AGM	10	CFU-Mix	18.0 ± 6.6	3.8 ± 3.3	4.8	0.001
		CFU-GM	16.7 ± 5.5	4.0 ± 4.7	4.2	0.022
		CFU-M	50.3 ± 10.6	12.0 ± 5.4	4.1	0.001
		BFU-E	29.0 ± 3.6	16.3 ± 7.4	1.8	0.042
		Total	130.3 ± 26.1	43.8 ± 19.6	3.0	0.004
PL		CFU-Mix	8.0 ± 7.1	1.6 ± 1.8	5.1	0.045
		CFU-M	19.7 ± 8.0	4.8 ± 4.2	4.1	0.002
		Total	165.5 ± 31.2	80.6 ± 38.8	2.1	0.004
YS		BFU-E	306.3 ± 113.4	183.6 ± 30.0	1.7	0.014
AGM	11	CFU-Mix	35.8 ± 14.2	19.4 ± 9.0	1.8	0.019
		CFU-M	80.6 ± 10.3	53.1 ± 9.4	1.5	0.006
		Total	199.4 ± 35.1	127.5 ± 20.5	1.6	0.002
FL		Total	1957.0 ± 691.0	1237.0 ± 348.0	1.6	0.046

The percentage of hypoxic cluster cells found in the E10 aorta correlates with the percentage decrease in CFU-C in the E10 AGM region, suggesting that only a subset of progenitor generation/function is controlled by the Hif1α pathway. These results are not due to incomplete recombination. PCR analysis of over 330 individual colonies revealed that 85–100% of CFU-C excised both *HIF1α*^{fl} alleles (Fig. 3). Moreover, cKO cells produce all colony types, and the morphology and size of the colonies were normal, suggesting that HIF1α does not affect progenitor function (proliferation or differentiation). Instead, our data suggest that the generation/expansion of HPCs in the *HIF1α*-deficient embryonic hematopoietic tissues is compromised in sites showing hypoxia.

Hematopoietic cells and clusters are quantitatively decreased in cKO embryos

To examine the hematopoietic cell content of *HIF1α* cKO embryonic tissues, flow cytometric analysis (FACS) was performed for c-Kit (expressed by all hematopoietic cluster cells), CD41 (expressed by some hematopoietic cluster cells), and CD31 (expressed by all endothelial and hematopoietic cluster cells) (Robin et al., 2011; Yokomizo and Dzierzak, 2010) (Table 2A). *HIF1α* deficient E10 AGM tissue was 4.7-fold ($p < 0.05$) and 1.4-fold decreased in the percentage of c-Kit$^+$ cells and CD41^{int} cells respectively, as compared to WT AGM tissues. A 3.6-fold decrease in CD31$^+$c-Kit$^+$ cells ($p < 0.005$) and 3.4-fold decrease in CD31$^+$CD41^{int} cells ($p < 0.05$) was found in cKO E10 PL, as compared to WT PL. At E11 a 1.3-fold decrease in PL c-Kit$^+$ cells, but no decrease in AGM c-Kit$^+$ cells and a slight decrease in AGM CD41$^+$ cells were found in cKO embryos. The E10 YS showed slight, but not significant decreases in c-Kit$^+$ and CD41$^+$ cells. These fold-decreases in phenotypic hematopoietic cells in cKO AGM, PL and YS correspond well to the quantitative changes observed for CFU-Cs.

We next examined whether intra-aortic hematopoietic cluster formation is affected by the loss of *HIF1α*. Three-dimensional imaging was performed on E10 whole mount +/+:*HIF-1α*$^{fl/fl}$ and *VEC-Cre*/+:*HIF-1α*$^{fl/fl}$ immunostained embryos (c-Kit and CD31) (Yokomizo and Dzierzak, 2010). As observed by the pattern of CD31 expression, the cKO vasculature appeared normal. However, a 1.2-fold decrease in the number of hematopoietic clusters and a 1.6-fold decrease in the number of CD31$^+$c-Kit$^+$ cells were found in cKO as compared to WT embryos (Table 2B). These data indicate that HPC/HSC formation from *HIF1α*-deficient hemogenic endothelium is

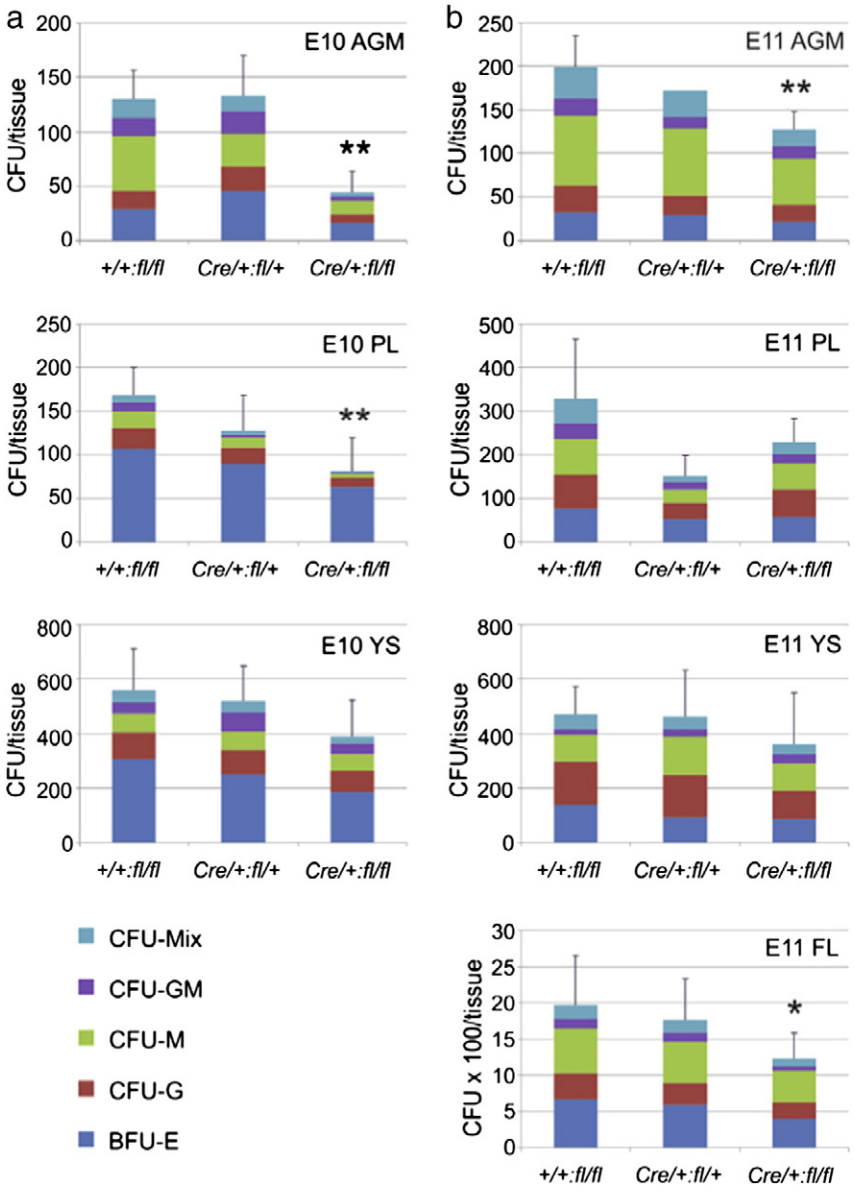
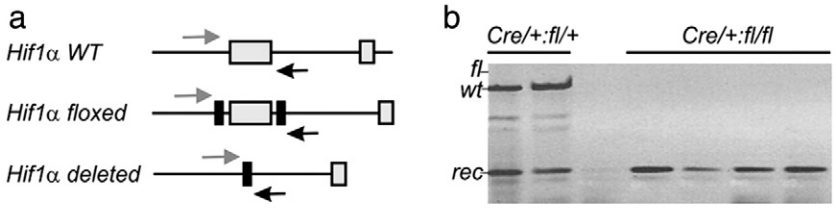


Figure 2 Hematopoietic progenitor numbers in *HIF1α* conditional deleted embryonic hematopoietic tissues. (a) Colony forming unit-culture (CFU-C) numbers per AGM, placenta (PL), and yolk sac (YS) in +/+:*HIF1α*$^{fl/fl}$, *VEC-Cre*/+:*HIF1α*$^{fl/+}$ and *VEC-Cre*/+:*HIF1α*$^{fl/fl}$ embryos at E10 (30–34 somite pair stage (sp)). (b) CFU-C numbers per AGM, PL, YS and FL at E11 (43–47sp). The colony numbers for each type (CFU-MIX, CFU-granulocyte, macrophage (GM), CFU-macrophage (M), CFU-granulocyte (G)) and BFU-E (Burst forming unit-erythroid) are shown as part of the total. Significant differences are indicated with asterisks (*: p-value ≤ 0.05, **: p-value ≤ 0.005). Data are the average CFU-C number per tissue derived from 4 to 7 embryos (3 separate litters) for each genotype (mean ± SD).

impaired and/or the *HIF1α*-deficient HPC/HSC fails to expand normally after emergence.

HIF1α-deficiency in VEC-expressing cells affects AGM and placenta long-term repopulating HSCs

To test whether long-term HSC repopulating ability of *VEC-Cre*/+:*HIF-1α*$^{fl/fl}$ hematopoietic tissues was affected, E11 AGM cells (1 or 2 embryo equivalents (ee)) or E11/E12 PL cells (1 or 0.3 ee) were injected into irradiated adult recipients and donor cell hematopoietic engraftment examined at greater than four months post-transplantation (Fig. 4a). Whereas 50% (3 out of 6) of recipients injected with WT AGM cells were high-level engrafted (90–95%), none of the 5 recipients receiving cKO AGM cells were repopulated to levels greater than 10%. Two cKO AGM recipients showed only low-level engraftment (1.2–7.4%). Similar percentages of mice were repopulated by cKO PL cells (4 out of 14) as compared to WT (4 out of 12). However, cKO PL cells yielded lower chimerism than WT PL cells (average 11% *versus* 28%, respectively) (Fig. 4b). Despite this lower level chimerism, cKO donor AGM



c

Stage	Genotype	number of deleted colonies/total number picked (%)			
		AGM	Placenta	Yolk Sac	Fetal Liver
E10	*Vec-Cre/+:HIF1α$^{fl/fl}$*	27/30 (90)	30/30 (100)	26/30 (87)	ND
	Vec-Cre/+:HIF1α$^{fl/+}$	20/20 (100)	19/20 (95)	18/20 (90)	ND
	+/+:HIF1α$^{fl/fl}$	0/5 (0)	0/5 (0)	0/5 (0)	ND
E11	*Vec-Cre/+:HIF1α$^{fl/fl}$*	36/40 (90)	35/40 (87)	36/40 (90)	36/40 (90)
	Vec-Cre/+:HIF1α$^{fl/+}$	19/20 (95)	20/20 (100)	17/20 (85)	18/20 (90)
	+/+:HIF1α$^{fl/fl}$	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

Figure 3 DNA PCR analysis for detection of the *HIF1α* allele in hematopoietic colonies. (a) Schematic of the *HIF1α* wild type, floxed and recombined alleles with PCR primers indicated. *LoxP* sites are shown as black rectangles. (b) Amplified DNA fragments resulting from PCR performed on cells isolated from single CFU-C of *VEC-Cre/+:HIF1α$^{fl/+}$* and *VEC-Cre/+:HIF1α$^{fl/fl}$* hematopoietic tissues. Fragments detected are *HIF1α* wild type (WT) and deleted (del) alleles. (c) Summary table of PCR results for 330 CFU-C obtained from E11 AGM, placenta, yolk sac and fetal liver cells of *VEC-Cre/+:HIF1α$^{fl/fl}$* and *VEC-Cre/+:HIF1α$^{fl/+}$* and *+/+:HIF1α$^{fl/fl}$* embryos.

and PL cells were found to give multi-tissue (blood, BM, spleen, thymus, lymph node) and multi-lineage (B and T lymphocytes, macrophage, granulocyte and erythroid cell) repopulation (Figs. 4c and d, middle panel). Engraftment was by fully deleted HSCs, as shown by PCR analysis. Whereas AGM HSCs provided no hematopoietic engraftment in secondary recipients, the bone marrow from one of the cKO placenta cell engrafted recipients (64% primary donor chimerism) showed self-renewing capability and high level chimerism in three transplanted irradiated adult recipients (Fig. 4d, right panel). Thus, the deletion of *HIF1α* during the generation of hematopoietic cells from embryonic vascular endothelial cells reduces the number and the quality of HSCs, supporting a role for HIF1α in some aspects of HSC development and function.

HIF1α is required for BM HSC maintenance in stress conditions

Despite the quantitative and qualitative decreases in hematopoietic progenitors and HSCs in cKO embryos, adult *VEC-Cre/+:HIF1α$^{fl/fl}$* mice without overt phenotypic defects were obtained. FACS analysis of cKO peripheral blood, spleen, lymph node and thymus cells (defined by expression of Gr-1, Mac-1, B220, CD4, CD5, TER119 and c-Kit) revealed no significant differences as compared to WT cells (not shown). HPC frequency in cKO BM, as assessed by CFU-C assay was similar to the frequency in WT BM (Fig. 5a). Only a slight (not significant) decrease in hematopoietic chimerism was observed with cKO cells in recipients when equal numbers of WT or cKO BM Lin$^-$c-Kit$^+$Sca1$^+$ (LSK) cells or unfractionated adult spleen cells were transplanted. WT LSK cells showed on average 48% PBL chimerism, whereas the recipients of cKO LSK showed on average 30% chimerism (Fig. 5b). Recipients of WT and cKO spleen cells showed 51% and 40% PBL chimerism, respectively (Fig. 5C). At six months post-transplantation, all hematopoietic tissues (BM, spleen, LN and thymus) showed donor cell engraftment levels comparable to PBL chimerism (not shown). However, FACS analysis showed a significant 2.4- to 3.6-fold reduction ($p < 0.05$) in the frequency of BM LSK cells in recipients of cKO LSKs, as compared to recipients of WT LSK cells (Fig. 5d). This finding is consistent with the result reported by Takubo et al. (2010) and indicates that HIF1α is essential for maintaining a normal level of LSK cells in stress conditions (*i.e.* transplantation).

Discussion

We demonstrate here for the first time that localized regions of hypoxia exist in the E10 aorta and placenta, as well as the liver at E11, and that the loss of *HIF1α* leads to decreases in AGM HPCs and HSCs. By deleting *HIF1α* specifically in embryonic endothelial cells, the precursors to emerging hematopoietic cluster cells, we have shown that HIF1α regulates HSCs and HPCs during and after their generation. Deletion of *HIF1α* leads to reductions in the number of HPCs, decreased frequencies of phenotypic HPC/HSCs, decreased aortic cluster numbers, and reductions in AGM and PL long-term repopulating HSCs. Adult BM *HIF1α* deficient HSCs show a sensitivity to the stress conditions of transplantation. Taken together, the HIF1α mediated response to hypoxia plays multiple roles in regulating HPCs and HSCs during development.

The embryonic vasculature, hematopoietic clusters and HPC emergence

The major embryonic vasculature is known to be the source of all definitive HPCs and HSCs, as shown by lineage tracing experiments with *VEC-Cre* induced marker recombination

Table 2 Percentages of phenotypic hematopoietic progenitor/stem cells in the tissues of +/+:*HIF1α*$^{fl/fl}$ and *VEC-Cre*/+:*HIF1α*$^{fl/fl}$ embryos. (A) Flow cytometric analysis performed on cells isolated from E10 (35–37sp) and E11 (>43sp) AGM (plus short segment of intrabody vitelline and umbilical arteries) and placenta (PL) and E10 (35–37sp) yolk sac (YS) tissues. The cellularity of hematopoietic tissues of *HIF1α* deficient embryos (AGM, PL and YS) was found to be the same as WT hematopoietic tissues (not shown). Cells were stained with antibodies specific for CD31, CD41 and c-Kit markers. The percentage marker positive cells within the viable single cell population are shown. Three to six tissues were analyzed (mean ± SD). Fold changes in percentage between WT and conditional knockout (cKO) tissues are shown and significant differences are indicated with asterisks (*p-value < 0.05; **p-value < 0.005). (B) Quantitation of the number of c-Kit positive cells and hematopoietic clusters in the aorta of whole mount imaged E10 +/+:*HIF1α*$^{fl/fl}$ (WT) and *VEC-Cre*/+:*HIF1α*$^{fl/fl}$ (cKO) embryos. The average number of c-Kit$^+$ cells per cluster (3–4) remained the same in the cKO aorta. (n = 2).

A

Tissue	FACS marker	Percentage positive cells		Fold decrease
		WT	cKO	
E10 AGM	c-Kit	5.6 ± 3.0	1.2 ± 3.0	4.7*
E10 AGM	CD41	1.0 ± 0.3	0.7 ± 0.2	1.4
E11 AGM	c-Kit	8.8 ± 0.8	9.8 ± 0.6	–
E11 AGM	CD41	2.5 ± 0.8	2.3 ± 0.4	1.1
E10 PL	c-Kit (in CD31$^+$)	43.4 ± 0.5	12.1 ± 4.0	3.6**
E10 PL	CD41 (in CD31$^+$)	24.0 ± 0.7	7.2 ± 1.2	3.3*
E11 PL	c-Kit (in CD31$^+$)	27.6 ± 1.4	20.8 ± 3.2	1.3*
E10 YS	c-Kit (in CD31$^+$)	4.5 ± 0.8	3.8 ± 0.5	1.2
E10 YS	CD41 (in CD31$^+$)	7.1 ± 1.2	5.0 ± 1.5	1.4

B

Whole mount embryo	Number of aortic clusters or cells			
	WT (33sp)	cKO (33sp)	cKO (32sp)	Average fold decrease
c-Kit$^+$ cells	231	182	139	1.4
clusters	66	42	38	1.6
Average c-Kit$^+$ cells/cluster	3	4	4	

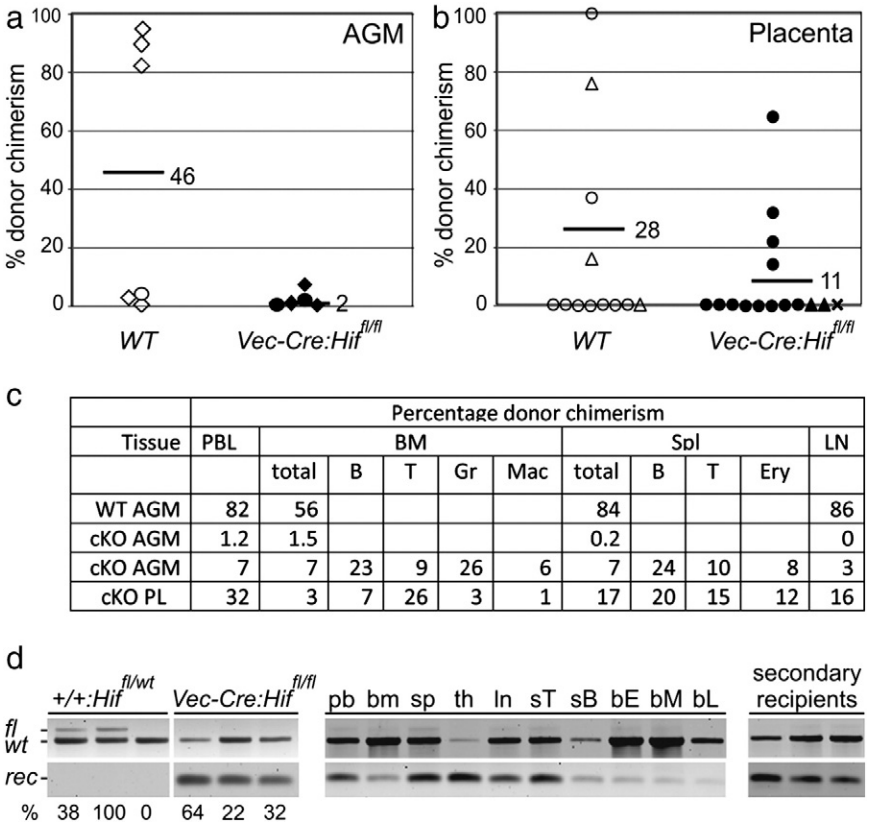
(Chen et al., 2009; Zovein et al., 2008). *VEC-Cre* expression and activity begin at about E7.5 in blood islands of yolk sac, chorionic mesoderm, and vitelline artery. At E9.5, *VEC-Cre* activity is observed in the aorta and umbilical artery. As HPCs and HSCs are first detected at E8.5 and E10.5 respectively in the aorta, vitelline and umbilical arteries, the loss of *HIF1α* is likely to affect their generation from the subset of hemogenic endothelial cells lining these vessels.

Interestingly, Hypoxyprobe staining revealed hypoxic endothelial cells and hematopoietic clusters lining the aorta at E10 at the same developmental time when a significant decrease in CFU-C numbers in the cKO AGM was found. This decrease in CFU-C numbers correlates with the hematopoietic clusters and cluster cell numbers found in the E10 aorta. Similarly, the E10 PL and E11 FL show an abundance of hypoxic endothelial and hematopoietic cells that temporally correlates with a significant decrease in CFU-C numbers in these tissues of cKO embryos. It was more difficult to determine if the E10 YS is hypoxic due to background staining. However, a significant decrease in only BFU-E and not for total CFU-C was found in E10 cKO YS (Table 1, lower panel). Hence the correlation between tissue specific hypoxia and the decrease in *HIF1α* cKO CFU-C numbers strongly supports a role for the HIF1α hypoxic response in the generation and expansion of some HPCs.

HIF1a regulates only some HSCs

A role for the HIF1α hypoxic response in HSC generation is clearly emphasized by the fact that long-term repopulating HSCs are profoundly decreased/absent in the E10 AGM tissue, but less so in the E11 cKO PL. Hypoxyprobe analysis showed highly hypoxic cells in the aorta (endothelial cells and hematopoietic clusters), but not all hematopoietic cluster cells or clusters were Hypoxyprobe positive. This, together with the fact that all methylcellulose colonies analyzed by PCR had both *HIF1α* floxed alleles deleted, shows that some HPC are *HIF1α*-independent. Similarly, PCR analysis of long-term recipients of AGM and PL cells showed multilineage hematopoietic engraftment. However, only PL cells could be serially transplanted. These data indicate that some HSCs are *HIF1α*-independent and suggest that whereas the AGM may be profoundly sensitive to hypoxia and loss of HIF1α, other hematopoietic tissues such as the PL are less so, and may act as the source of the adult repopulating HSCs found in the adult BM.

Whole embryo confocal imaging also shows clear quantitative decreases in vascular hematopoietic clusters, suggesting that either the endothelial-to-hematopoietic transition is affected or the growth/expansion of the emerging HPCs and HSCs in the clusters is affected. Since the receptor tyrosine



	Percentage donor chimerism										
Tissue	PBL	BM					Spl				LN
		total	B	T	Gr	Mac	total	B	T	Ery	
WT AGM	82	56					84				86
cKO AGM	1.2	1.5					0.2				0
cKO AGM	7	7	23	9	26	6	7	24	10	8	3
cKO PL	32	3	7	26	3	1	17	20	15	12	16

Figure 4 *In vivo* long-term hematopoietic transplantation analysis of *HIF1α* cKO AGM and placenta cells. Peripheral blood chimerism of adult irradiated recipients injected with wild type (WT) and *VEC-Cre/+:HIF1α*$^{fl/fl}$ (a) AGM and (b) placenta cells was assessed by FACS analysis for donor Ly5.2 hematopoietic cells at greater than 5 months post-transplantation. (a) Percentage engraftment in recipients injected with 2 ee of AGM cells from WT (open diamond) and cKO (black diamond), or 1 ee of AGM cells from WT (open circles) and cKO (black circles) E11 (46–50sp stage) embryos (n = 2). (b) Percentage engraftment of recipients injected with 0.3 ee of E12 placenta cells from WT (open triangle) and cKO (black triangle), 1 ee of E12 cKO placenta cells (cross), or 1 ee of E11 placenta cells from WT (open circles) and cKO (black circles) embryos (n = 3). Average percentage donor cell chimerism for each transplantation group is shown next to the line. (c) Multilineage engraftment analysis of three recipients of *VEC-Cre/+:HIF1α*$^{fl/fl}$ E11 AGM and placenta (PL) cells and one WT control. Recipients were sacrificed at greater than 5 months post-injection and donor cell chimerism was assessed by FACS analysis for the donor cell Ly5.2 marker in peripheral blood leukocytes (PBL), bone marrow (BM), spleen (Spl) and lymph nodes (LN) and in specific lineages — B lymphoid (B), T lymphoid (T), granulocyte (G), macrophage (M) and erythroid (Ery). Percentages of donor cells in each tissue/lineage are shown. (d) PCR analysis to test for deletion of *HIF1α* the donor-derived engrafted cells. PCR analysis was performed on peripheral blood leukocyte DNA from 3 recipients transplanted with *+/+:HIF1α*$^{fl/fl}$ and 3 recipients transplanted with *VEC-Cre/+:HIF1α*$^{fl/fl}$ E11 placenta cells (see panel B) at 8 months post-injection. WT cell engraftment (38% and 100%) is observed by the presence of the 1 kb *HIF1α fl* fragment. The presence of the recombined *HIF1α* (0.3 kb) fragment and indicate engraftment levels of 64%, 22% and 32% with cKO placenta cells. The wt *HIF1α* fragment (0.96 kb) is derived from recipient cells. All engraftment levels were consistent with the donor cell Ly5.2 marker analysis. (Middle panel) multilineage PCR analysis of peripheral blood leukocytes (pb), bone marrow (bm), spleen (sp), thymus (th), lymph node cells (ln), spleen T, B lymphocytes (sT, sB) and bone marrow erythroid, myeloid and lymphoid cells (bE, bM, bL) from the 22% engrafted recipient injected with *VEC-Cre/+:HIF1α*$^{fl/fl}$ E11 placenta cells at 8 months post-transplantation. The recombined *HIF1α* fragment but not the *fl HIF1α* fragment is visible in each lane. (Right panel) PCR analysis of three secondary recipients at 4 months postinjection of (3 × 106) bone marrow cells from primary engrafted (64%) recipient.

kinase Flk1 is known to be expressed on endothelial cells of the midgestation vasculature and moreover, on emerging aortic hematopoietic cluster cells (Yokomizo and Dzierzak, 2010) and *VEGF* is a direct target of HIF1α (Liu et al., 1995), it is possible that the activation of this signaling pathway in the embryonic vasculature (Lee et al., 2001) may influence EHT in the hypoxic embryonic endothelial cells. In addition, a recent study in Zebrafish embryos indicates that metabolism affects AGM HSC formation (Harris et al., 2013). Growth of embryos in high glucose increased HSC numbers through a HIF1α mediated response. A knockdown of HIF1α in embryos showed a decrease in runx1/cMyb positive cells. These data are in line with our findings that HIF1α is required for HSC formation in the dorsal aorta and highlight the evolutionary conservation of this pathway through vertebrate development. Future studies will examine the expression of such molecules, as well as other downstream targets of HIF1α in the context of endothelial-to-hematopoietic transition.

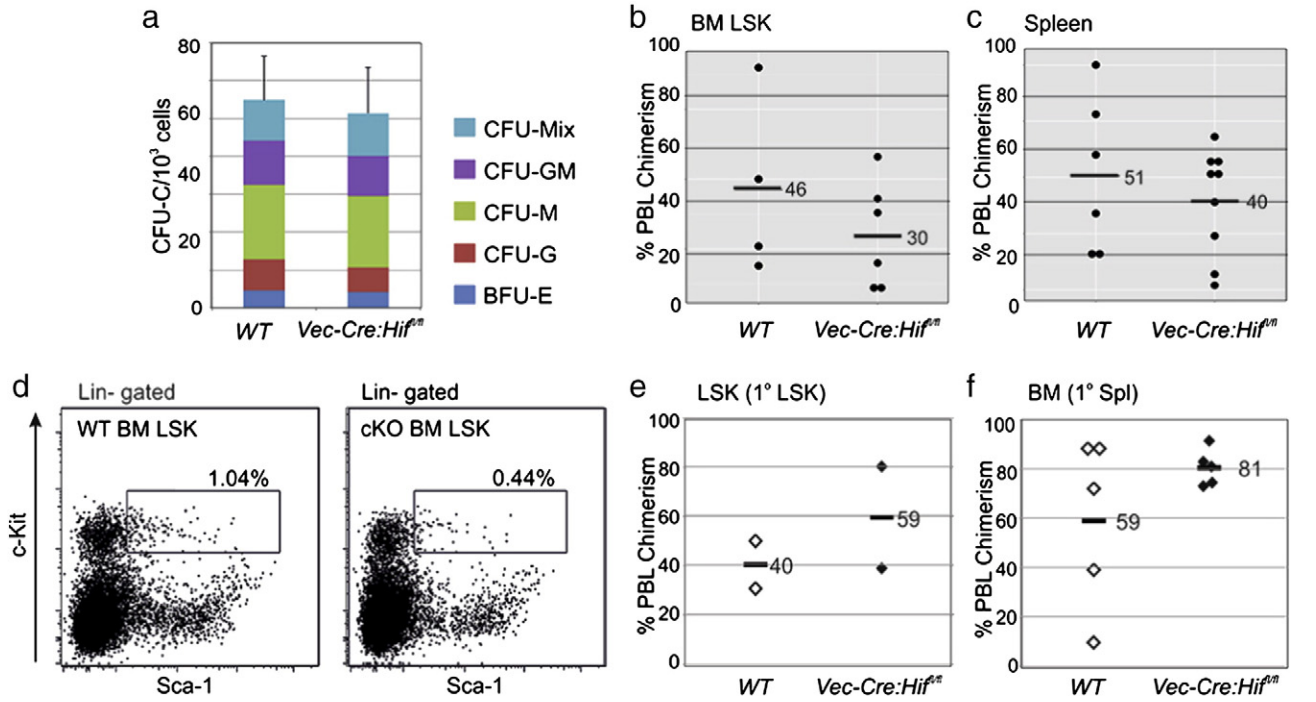


Figure 5 *HIF1α* is required for the maintenance of adult HSCs under transplantation stress conditions. (a) CFU-C frequency in the bone marrow cells of adult +/+:*HIF1α*$^{fl/fl}$ and *VEC-Cre*/+:*HIF1α*$^{fl/fl}$ mice. 3 × 10^4 bone marrow cells from WT and cKO mice were cultured in methylcellulose medium in triplicate. The CFU-C data is averaged (mean ± SD) from 3 WT and 4 cKO mice. (b) Hematopoietic stem cell repopulating potential of *VEC-Cre*/+:*HIF1α*$^{fl/fl}$ bone marrow LSK cells. 1500 sorted LSK cells isolated from two +/+:*HIF1α*$^{fl/fl}$ and three *VEC-Cre*/+:*HIF1α*$^{fl/fl}$ adult male mice were injected into 4 and 6 female recipients, respectively. (c) Hematopoietic stem cell repopulating potential of *VEC-Cre*/+:*HIF1α*$^{fl/fl}$ spleen. 2 × 10^6 cells from WT and cKO spleens were injected into 6 and 9 female recipients, respectively. (d) LSK cell flow cytometric analysis of bone marrow from primary recipients of +/+:*HIF1α*$^{fl/fl}$ and *VEC-Cre*/+:*HIF1α*$^{fl/fl}$ BM LSK cells at 6 months post-transplantation. A representative scatter plot for c-Kit and Sca-1 expression on gated Lin$^-$ cells for each genotype is shown. Efficiency of recombination for the *HIF1α*$^{fl/fl}$ alleles of recipients was found to be 85–100%. (e and f) Secondary transplantations were performed with (e) 600 sorted bone marrow LSK cells from primary recipients of bone marrow LSK cells of WT and cKO adult mice with similar levels of primary engraftment or (f) 2 × 10^6 Ficoll fractionated bone marrow cells from primary recipients of spleen cells of WT and cKO adult mice (with similar level of primary engraftment) at 6 months post-transplantation. Percentage of peripheral blood leukocyte (PBL) chimerism in secondary recipients was assessed by Ly5.1/Ly5.2 flow cytometric analysis at FACS 4 months post-secondary transplantation. Efficiency of recombination for the *HIF1α*$^{fl/fl}$ alleles of recipients was found to be 46–93% for donor cells.

HIF1α in adult BM HSCs in stress conditions

Our analysis of cKO adult mice showed that HIF1α also plays a role in regulating BM HSC function under stress conditions. Steady state hematopoiesis in adult cKO mice appeared normal. The effects of *HIF1α* deficiency were only revealed upon transplantation and examination of the BM of the primary recipients — BM HSC content (LSK cells) was significantly decreased. It has been suggested that quiescent HSCs are maintained in a hypoxic niche at a lower oxidative stress to prevent their differentiation and exhaustion. They utilize glycolytic metabolism instead of mitochondrial oxidative phosphorylation to meet energy demands and show HIF1α up-regulation (Simsek et al., 2010). Studies of adult *MX1-Cre:HIF1α*$^{fl/fl}$ mice revealed that HIF1α (but not HIF2α Guitart et al., 2013) is required to maintain normal number of HSCs. Under stress condition such as serial transplantations, *HIF1α* deficient HSCs lose their quiescence (Takubo et al., 2010).

Secondary transplantations were performed to test the self-renewal capacity of *VEC-Cre*/+:*HIF1α*$^{fl/fl}$ cKO HSCs. LSK cells from primary recipients (WT and cKO LSK cells) with similar level of chimerism were injected into irradiated secondary recipients. Surprisingly, secondary recipients of cKO LSK cells showed a high chimerism levels at greater than 4 months post-injection (Figs. 5e and f), indicating that cKO BM HSCs self-renew. Recombination efficiency was approximated to be between 46 and 93% in most of the recipients. We thought that a change in the cell cycle status of BM LSK cells may occur. Indeed, our preliminary observations show that cKO LSK cells undergo unscheduled cell cycle entry and proliferation suggesting a role for HIF1α in cell cycle regulation of HSCs or HPCs under transplantation stress conditions. To verify this observation, more precise studies examining the competition between WT and cKO HSCs need to be performed with further markers and appropriate cell fractionations. Moreover, in future experiments WT HSCs will be transplanted into adult

VEC-Cre/+:HIF-1α$^{fl/fl}$ mice to examine the role of HIF1α in the adult BM microenvironment.

In conclusion, HIF1α plays an essential role in HPC and HSC generation in hypoxic sites of the developing embryo, particularly in the hemogenic endothelial stage in the AGM, as well as the fetal placenta and liver (although to a lesser degree). Understanding the precise role of HIF1α and physiological weak oxygenation, or "*in situ* normoxia," in regulating the endothelial-to-hematopoietic transition can provide new insights into *in vitro* manipulation and culture conditions that may allow the efficient *de novo* generation and/or expansion of HSCs for research and clinical applications.

Conflict of interest

The authors declare no competing financial or personal interests. P Imanirad, P Solaimani-Kartalei, M Crisan, C Vink, T Yamada-Inagawa, E de Pater, D Kurek and P Kaimakis performed research. R van der Linden collected data. P Imanirad, P Solaimani Kartalaei, M Crisan and E Dzierzak designed experiments, analyzed and interpreted data. N Speck contributed a new reagent. P Imanirad and E Dzierzak wrote the manuscript.

Acknowledgments

We thank Dies Meijer for microscopy expertise and the following funding agencies for support of these studies: Landsteiner Society for Blood Research (LSBR 1109), ZonMW (Dutch Medical Research Council (911-09-036 and 016.126.088)), EMBO Long-Term Fellowship (ALTF 260-2009), FES NIRM (Dutch Innovation Grant) and NIH (RO37 DK54077).

References

Adelman, D.M., Maltepe, E., Simon, M.C., 1999. Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. Genes Dev. 13, 2478–2483.

Boisset, J.C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., Robin, C., 2010. *In vivo* imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature 464, 116–120.

Chen, M.J., Yokomizo, T., Zeigler, B.M., Dzierzak, E., Speck, N.A., 2009. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature 457, 887–891.

Chow, D.C., Wenning, L.A., Miller, W.M., Papoutsakis, E.T., 2001. Modeling pO(2) distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. Biophys. J. 81, 685–696.

Cowden Dahl, K.D., Fryer, B.H., Mack, F.A., Compernolle, V., Maltepe, E., Adelman, D.M., Carmeliet, P., Simon, M.C., 2005. Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. Mol. Cell. Biol. 25, 10479–10491.

Danet, G.H., Pan, Y., Luongo, J.L., Bonnet, D.A., Simon, M.C., 2003. Expansion of human SCID-repopulating cells under hypoxic conditions. J. Clin. Invest. 112, 126–135.

de Bruijn, M.F., Speck, N.A., Peeters, M.C., Dzierzak, E., 2000. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. EMBO J. 19, 2465–2474.

Dunwoodie, S.L., 2009. The role of hypoxia in development of the mammalian embryo. Dev. Cell 17, 755–773.

Guitart, A.V., Subramani, C., Armesilla-Diaz, A., Smith, G., Sepulveda, C., Gezer, D., Vukovic, M., Dunn, K., Pollard, P., Holyoake, T.L., et al., 2013. Hif-2α is not essential for cell-autonomous hematopoietic stem cell maintenance. Blood 122, 1741–1745.

Harris, J.M., Esain, V., Frechette, G.M., Harris, L.J., Cox, A.G., Cortes, M., Garnaas, M.K., Carroll, K.J., Cutting, C.C., Khan, T., et al., 2013. Glucose metabolism impacts the spatiotemporal onset and magnitude of HSC induction *in vivo*. Blood 121, 2483–2493.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y., et al., 1998. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev. 12, 149–162.

Keith, B., Johnson, R.S., Simon, M.C., 2012. HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. Nat. Rev. Cancer 12, 9–22.

Kotch, L.E., Iyer, N.V., Laughner, E., Semenza, G.L., 1999. Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. Dev. Biol. 209, 254–267.

Kubota, Y., Takubo, K., Suda, T., 2008. Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche. Biochem. Biophys. Res. Commun. 366, 335–339.

Lee, Y.M., Jeong, C.H., Koo, S.Y., Son, M.J., Song, H.S., Bae, S.K., Raleigh, J.A., Chung, H.Y., Yoo, M.A., Kim, K.W., 2001. Determination of hypoxic region by hypoxia marker in developing mouse embryos *in vivo*: a possible signal for vessel development. Dev. Dyn. 220, 175–186.

Liu, Y., Cox, S.R., Morita, T., Kourembanas, S., 1995. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5′ enhancer. Circ. Res. 77, 638–643.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., Simon, M.C., 1997. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature 386, 403–407.

Medvinsky, A., Dzierzak, E., 1996. Definitive hematopoiesis is autonomously initiated by the AGM region. Cell 86, 897–906.

Mohyeldin, A., Garzon-Muvdi, T., Quinones-Hinojosa, A., 2010. Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell 7, 150–161.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., Down, J.D., 2007. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc. Natl. Acad. Sci. U. S. A. 104, 5431–5436.

Ramirez-Bergeron, D.L., Runge, A., Adelman, D.M., Gohil, M., Simon, M.C., 2006. HIF-dependent hematopoietic factors regulate the development of the embryonic vasculature. Dev. Cell 11, 81–92.

Raval, R.R., Lau, K.W., Tran, M.G., Sowter, H.M., Mandriota, S.J., Li, J.L., Pugh, C.W., Maxwell, P.H., Harris, A.L., Ratcliffe, P.J., 2005. Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel–Lindau-associated renal cell carcinoma. Mol. Cell. Biol. 25, 5675–5686.

Robin, C., Dzierzak, E., 2005. Hematopoietic stem cell enrichment from the AGM region of the mouse embryo. Methods Mol. Med. 105, 257–272.

Robin, C., Ottersbach, K., Boisset, J.C., Oziemlak, A., Dzierzak, E., 2011. CD41 is developmentally regulated and differentially expressed on mouse hematopoietic stem cells. Blood 117, 5088–5091.

Ryan, H.E., Lo, J., Johnson, R.S., 1998. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J. 17, 3005–3015.

Semenza, G.L., 2012. Hypoxia-inducible factors in physiology and medicine. Cell 148, 399–408.

Simon, M.C., Keith, B., 2008. The role of oxygen availability in embryonic development and stem cell function. Nat. Rev. Mol. Cell Biol. 9, 285–296.

Simsek, T., Kocabas, F., Zheng, J., Deberardinis, R.J., Mahmoud, A.I., Olson, E.N., Schneider, J.W., Zhang, C.C., Sadek, H.A., 2010. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. Cell Stem Cell 7, 380–390.

Takubo, K., Goda, N., Yamada, W., Iriuchishima, H., Ikeda, E., Kubota, Y., Shima, H., Johnson, R.S., Hirao, A., Suematsu, M., et al., 2010. Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. Cell Stem Cell 7, 391–402.

Wiesener, M.S., Jurgensen, J.S., Rosenberger, C., Scholze, C.K., Horstrup, J.H., Warnecke, C., Mandriota, S., Bechmann, I., Frei, U.A., Pugh, C.W., et al., 2003. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J. 17, 271–273.

Yokomizo, T., Dzierzak, E., 2010. Three-dimensional cartography of hematopoietic clusters in the vasculature of whole mouse embryos. Development 137, 3651–3661.

Yokomizo, T., Yamada-Inagawa, T., Yzaguirre, A.D., Chen, M.J., Speck, N.A., Dzierzak, E., 2012. Whole-mount three-dimensional imaging of internally localized immunostained cells within mouse embryos. Nat. Protoc. 7, 421–431.

Yoon, D., Pastore, Y.D., Divoky, V., Liu, E., Mlodnicka, A.E., Rainey, K., Ponka, P., Semenza, G.L., Schumacher, A., Prchal, J.T., 2006. Hypoxia-inducible factor-1 deficiency results in dysregulated erythropoiesis signaling and iron homeostasis in mouse development. J. Biol. Chem. 281, 25703–25711.

Zovein, A.C., Hofmann, J.J., Lynch, M., French, W.J., Turlo, K.A., Yang, Y., Becker, M.S., Zanetta, L., Dejana, E., Gasson, J.C., et al., 2008. Fate tracing reveals the endothelial origin of hematopoietic stem cells. Cell Stem Cell 3, 625–636.